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COMMUNICATION

Flaxseed Treatments to Reduce Biohydrogenation of α-Linolenic Acid by Rumen Microbes in Cattle

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Abstract Enrichment of beef muscle with n-3 fatty acids (FA) is one means to introduce these FA into the diet, but ruminal biohydrogenation limits their bioavailability. To address this problem, we evaluated the ability of condensed tannin (quebracho), in the presence or absence of casein, to protect 18:3n-3 in flaxseed from hydrogenation by ruminal microbes in cattle using an in vitro fermentation approach coupled with evaluation in cattle in vivo. Treated and untreated flaxseed was incubated with bovine rumen fluid for 0 and 24 h. With tannin treated flaxseed, hydrogenation of 18:3n-3 was limited to only 13% over 24 h compared to 43% for untreated flaxseed, while addition of casein to the tannin added no additional protection. To determine if a similar level of protection would occur in vivo, we used two groups of five steers fed either a grain-based or forage-based diet. Five steers were given a grain-based diet during the trial and were fed either ground flaxseed or tannin treated flaxseed for 15 days prior to blood collection for plasma lipid fatty acid analysis. The forage fed steers followed the same regimen. Ingestion of tannin-treated flaxseed did not increase 18:3n-3 and 20:5n-3 in plasma neutral lipids as compared to non-treated flaxseed. Thus, we demonstrated that treating ground flaxseed with quebracho tannin is not useful for increasing 18:3n-3 in the neutral lipid of bovine blood plasma, and suggest caution when interpreting results from in vitro trials that test potential treatments for protecting fatty acids from hydrogenation by ruminal microbes.

Keywords n-3 Fatty acid · Linseed · Protection · Bovine · Biohydrogenation

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Introduction

Although many people would benefit from consuming more n-3 fatty acids (FA) in their diets [1, 2], many individuals do not consume enough fish to obtain sufficient dietary intake of n-3 FA [3]. The sustainability of wild caught and farmed fish such as salmon is questionable [4, 5], suggesting additional n-3 FA sources are needed. Many people regularly consume red meat [6], however cattle and lambs are often fed diets high in grain before they are slaughtered producing meat that has very low n-3 FA levels [7]. If cattle or sheep consume immature forage or receive some flaxseed or flaxseed oil as part of their diet, they consume α-linolenic acid (18:3n-3) and a little of this reaches the small intestine, but most is hydrogenated by ruminal microbes [8]. Although this method produces meat enriched in n-3 fatty acids [9, 10], a production method that would provide additional pass through n-3 FA, thereby further enriching the meat and providing additional feed efficiency is needed.

It is clear that bypassing the rumen increases n-3 FA in plasma and muscle, hence a key element to using feedstuffs containing n-3 FA is to protect it in the rumen from biohydrogenation. The concentration of 18:3n-3 increases in the blood when emulsions of flaxseed oil are infused into the abomasum, the true stomach of ruminants, [11, 12] or into the duodenum, [13]. There is evidence that the 18:3n-3 levels of neutral lipid in the muscle of cattle and sheep can be increased if more 18:3n-3 is made available for absorption from the small intestine [14–16]. Protecting n-3 FA by encapsulating them in a matrix of formaldehyde-treated protein increases n-3 FA levels in the muscle [17]. Although this method is believed to provide protection for polyunsaturated FA (PUFA) from microbial hydrogenation [12, 18], the use of formaldehyde lowers its consumer acceptance. In addition, there are conflicting results with respect to the effectiveness of this process [19, 20]. Several other approaches have been tested for protecting n-3 FA from microbial hydrogenation in the rumen that do not use formaldehyde, but these have had little success [20–24]. More recently, a method using a whey protein isolate gel appears promising [25, 26], but this approach may be impractical.

Therefore, the primary objective of this study was to find a practical, simple, and consumer-acceptable method to protect the 18:3n-3 in flaxseed from hydrogenation by rumen microbes. Condensed tannin has the ability to bind with plant proteins in the rumen, with enzymes secreted by rumen bacteria, and with rumen bacteria, but do this without altering absorption in the entire gastrointestinal tract [27, 28]. Hence, we rationalized that tannins might help protect 18:3n-3 in flaxseed from hydrogenation by rumen microbes. Furthermore, we rationalized that adding the protein casein to ground flaxseed before treating it with condensed tannin may help encase the 18:3n-3 in flaxseed in a protective coating. Therefore, the specific objectives were to: (1) determine if treating ground flaxseed with tannin with or without casein would result in less disappearance of 18:3n-3 during fermentations in vitro with cattle rumen fluid, and (2) determine if treating ground flaxseed with condensed tannin could increase the level of 18:3n-3 and other n-3 FA in the blood of cattle consuming forage or grain-based diets.

Experimental Procedures

Flaxseed Treatment with Tannin

Ground flaxseed was treated with condensed tannin (quebracho tannin), or condensed tannin plus either low (45 g/227 g flaxseed) or high (90.8 g/227 g flaxseed) amounts of casein. Ground flaxseed was treated with condensed tannin by mixing 45.4 g of quebracho tannin syrup (34% DM; Tannin Corp., Peabody, MA) with 28.3 g of warm tap water then thoroughly mixing this solution with 227 g of ground flaxseed before drying in a forced air oven at 50 °C. For flaxseed containing casein, either 45.4 g of casein (Sigma-Aldrich, St. Louis, MO) or 90.8 g of casein was mixed with 227 g of ground flaxseed to which a solution containing 45.4 g of quebracho tannin syrup in 125 g of warm tap water was added and thoroughly mixed. The flaxseed-tannin-casein mixtures were dried as described above for the flaxseed-tannin mixture.

Fermentation In Vitro

Fermentation of untreated and treated flaxseed with bovine rumen fluid was done at 39 °C and the reduction in 18:3n-3 assessed at 24 h. Each flask contained 250 mg of ground alfalfa-grass hay, 250 mg of untreated ground flaxseed or one of three treated flaxseeds, 40 ml of buffer solution, and 10 ml of strained rumen fluid. Rumen fluid was obtained from two ruminally fistulated beef cows and kept separate in pre-warmed insulated containers until it was used in the trial within 30 min after collection. The cows were fed alfalfa hay ad libitum for a month before ruminal digesta was collected for the trial. The rumen fluid was flooded with CO_2 while it was put into the flasks and again after the addition of the feedstuffs. Flasks were agitated at 1, 12 and 18 h. Microbial activity in the 0 time samples as well as 24 h fermentations was stopped by pouring the contents of each flask into a plastic bag then quickly freezing it as a thin (ca. 0.5 cm) layer. The buffer solution was distilled water and 0.68 mM $CaCl_2$, 2 mM $MgSO_4$, 73 mM KH_2PO_4, 142 mM Na_2CO_3, 8.6 mM $NaCl_2$, 7.9 mM Na_2SO_3, and 8.3 mM NH_2CONH_2. Each combination of flaxseed treatments was replicated twice with rumen fluid from one cow used in one replicate set and rumen fluid from the second cow used in the other.

Calculations for the Fermentation Reaction

The percent change in the 18:3n-3 level at 24 h was determined relative to the initial levels at time 0. Percent change was used because initial amount of this FA varied somewhat as a function of whether or not the flaxseed was or was not treated with tannin. The Mixed Model Procedure of SAS [29] was used for data analysis, and tests were conducted to assure that data were normally distributed. The rumen-fluid donor cow was the random factor in all statistical analyses. The variance-covariance structure was examined and the variance components option was used. Means were considered different at an α of 0.05.

Assessment of Tannin-Treated Flaxseed In Vivo

Ten yearling Angus steers (castrated males) with a mean body weight of 392 kg (SD = 21 kg) were used in a trial with a factorial design with flaxseed treatment (none or tannin-treated) and time of blood collection (pre- or post-prandial) as the two factors. Separate analyses were conducted for each type of basal diet, which were either grain or forage. Five of the steers were given a grain-based diet during the whole trial and were given tannin-treated ground flaxseed for 15 days followed by collection of blood into heparinized tubes. These cattle were then fed non-treated ground flaxseed for 15 days before blood was collected. The other five steers on the forage-based diet followed the same treatment regimen as steers on the grain-based diet.

The steers were fed at 6 am and 6 pm, and on average had a daily intake of 7.8 or 8.8 kg of the grain or forage basal rations, respectively, plus 907 g of either ground flaxseed or 980 g of tannin-treated ground flaxseed (half of each fed per feeding). More tannin-treated flaxseed was fed to adjust for the additional weight of the tannin so that cattle received the same amount of flaxseed. Ground flaxseed was treated with quebracho tannin by mixing the flaxseed meal with tannin and water at a rate of 5:1:0.625 (w/w/w) then drying in a forced-air oven at 50 °C. Both grain and forage rations were formulated to meet or exceed the nutrient requirements of the growing steers [30].

The feed and nutritional composition of the two basal diets is described in Table 1 along with their composition of 18-carbon FA. Liquid molasses was added to the grain mixture to help maintain its integrity when fed, improve its palatability, and reduce feed dust. The alfalfa hay cubes in the forage-based diet were about 4 cm^3 in size and composed of coarsely chopped alfalfa hay. The same amount of liquid molasses and mineral-vitamin mixture was added to the forage ration.

Plasma neutral and phospholipid n-3 FA levels were evaluated with respect to flaxseed treatment and time of blood collection (post-prandial, 10-30 to 11 am or pre-prandial, 5-30 to 6 pm) using the Mixed Model Procedure of SAS [29]. Separate analyses were done for animals on the two basal diets since their daily intake of 18:3n-3 from the basal diets was 14 g for the forage-fed steers versus 8 g for the grain-fed steers. Animals were the random factor for both analyses. The variance-covariance structures were examined and the variance components option was used for both analyses. Means were considered different at an α of 0.05.

Plasma Total Lipid Extraction and Fatty Acid Analysis

Plasma lipids were then extracted by transferring a 200 µl aliquot of plasma into a tube containing 5 ml of chloroform-methanol (2:1, v/v) followed by mixing it by vortexing [31]. The addition of 1 ml of 0.9% KCl to these tubes resulted in two phases, which were thoroughly mixed and then separated overnight in a −20 °C freezer. The upper phase was removed, and the lipid-containing lower phase was rinsed with 1 ml of theoretical upper phase, consisting of chloroform-methanol-water (3:48:47, v/v), to remove any aqueous soluble contaminants [31]. The upper phase was discarded and the neutral lipids and phospholipids separated using solid-phase extraction with silicic acid [10]. The neutral lipids were eluted with chloroform and the phospholipids eluted with methanol. The respective solvents were removed via evaporation under a stream of nitrogen and the lipids redissolved in *n*-hexane:2-propanol (3:2 v/v). This lipid extract was stored at −80 °C until analysis.

A portion of each lipid fraction was used to determine fatty acid composition of the plasma neutral lipids and phospholipids. The *n*-hexane:2-propanol was removed by evaporation under a stream of nitrogen and 2 ml of 0.5 M KOH dissolved in anhydrous methanol was added to make fatty acid methyl esters (FAME) via a base-catalyzed transesterification [32]. The FAME were extracted from the methanol using 2 ml of *n*-hexane and the *n*-hexane phase containing the FAME was removed. The lower phase was reextracted two more times with 3 ml of *n*-hexane, and these washes were combined with the original aliquot. Individual FA were separated by GLC using an SP-2330 column (0.32 mm i.d. × 30 m length) and a Trace GLC (ThermoElectron, Austin, TX) equipped with dual auto-samplers and dual FID. FA were quantified using a

Table 1 Ingredients and nutrient composition of the grain and forage basal diets consumed by yearling beef steers

	Grain	Forage
Ingredient (% as fed)		
Rolled corn	58	–
Cracked peas	6	–
Soybean meal	3	–
Sugar beet pulp pellets	25	50
Alfalfa cubes	–	42
Liquid molasses	7	7
Mineral-vitamin mixture[a]	0.7	0.7
Composition (% of DM)		
Nitrogen	1.59	2.09
IVDMD	79	77
Fatty acids		
18:0	0.08	0.04
18:1n-9	1.12	0.18
18:2n-6	2.61	0.38
18:3n-3	0.10	0.16

[a] Mineral-vitamin mixture = dicalcium phosphate, trace mineral salt, limestone, and vitamin A, D, and E

standard curve from commercially purchased standards (Nu-Chek-Prep, Elysian, MN) and 17:0 was used as the internal standard.

Fatty Acid Analysis of Feed for Cattle Trial and In Vitro Residue

Dietary ingredients were analyzed for fatty acids via direct transesterification with methanolic-HCl [33, 34]. In vitro fermentation samples were lyophilized (Freezemobile 25 SL, The Virtis Co., Gardiner, NY), ground with a mortar and pestle, and analyzed for fatty acids [35]. Briefly, 200 mg was subjected to direct saponification in 4.0 ml of ethanol containing 1 ml of 33% (w/v) KOH. Direct saponification was done in 16 × 25 mm tubes with Teflon-lined screw-caps at 85 °C for 1 h with vortex-mixing every 1 min. Tubes were cooled, and 1.0 ml of 12 M HCl and 3.0 ml of hexane were added to each tube and mixed by vortexing. The hexane layer was transferred to a clean tube and dried under a stream of N_2 gas. Fatty acid methyl esters were prepared by incubating the dried hexane layer with 4.0 ml of 0.5 M HCl in methanol that contained 1 mg of tridecanoic acid (Sigma, St. Louis, MO) as the internal standard for 1 h at 85 °C. Separation of fatty acid methyl esters was achieved by GLC (Model CP-3800, Varian Inc., Palo Alto, CA) equipped with a 100 m capillary column (SP-2560, Supelco, Bellefonte, PA). Identification of peaks was accomplished using purified standards (Sigma-Aldrich, St. Louis, MO; Nu-Chek Prep, Elysian, MN).

Results

In Vitro Fermentation Trial

The percent change in level of 18:3n-3 at 24 h were similar ($P = 0.73$) among the three tannin treatments, indicating that adding either of the two amounts of casein to the flaxseed-tannin mixture did not reduce hydrogenation (Fig. 1). However, across the 24-h period, treating the ground flaxseed with condensed tannin reduced ($P = 0.01$) hydrogenation of 18:3n-3 to only 13% compared to a loss of 43% in the untreated flaxseed.

Assessment of Tannin-Treated Flaxseed In Vivo

With respect to neutral lipids in blood plasma, when the basal diet was either forage or grain, ingesting tannin-treated flaxseed did not raise plasma 18:3n-3 levels over those observed when the cattle consumed normal ground flaxseed ($P \geq 0.10$; Fig. 2). Levels of 20:5n-3 were not raised by ingestion of treated flaxseed regardless of basal diet ($P \geq 0.17$) nor were total n-3 levels (18:3n-3 and 20:5n-3) for either basal diet ($P \geq 0.07$).

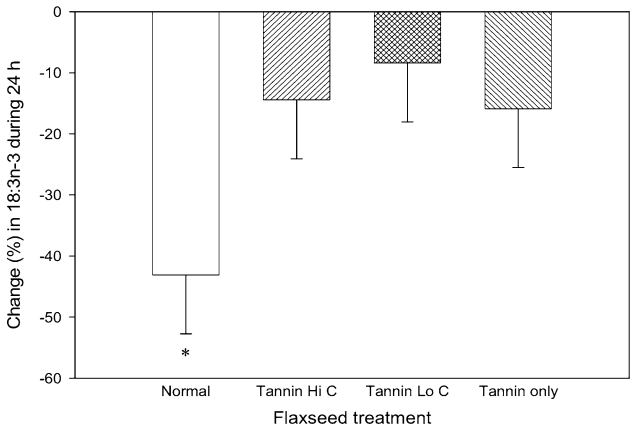

Fig. 1 Least square means and standard errors (*T-bars*) for % change in 18:3n-3 during the 24 h period of fermentations in vitro of normal ground flaxseed (*Normal*), ground flaxseed mixed with a high amount of casein then treated with condensed tannin (*Tannin Hi C*), ground flaxseed mixed with a low amount of casein then treated with condensed tannin (*Tannin Lo C*), or ground flaxseed treated with only condensed tannin (*Tannin only*). The *asterisk* indicates significance from control $P < 0.05$

The level of plasma neutral lipid 18:3n-3 was similar at both blood collection times for forage-fed steers ($P = 0.38$), but 18:3n-3 level was slightly higher (4.8%) at the pre-prandial collection time for the grain-fed steers ($P = 0.04$). Neutral lipid 20:5n-3 level did not differ as a function of time of blood collection for either the forage- or grain-fed steers ($P \geq 0.50$).

The ingestion of tannin-treated flaxseed increased blood plasma phospholipid 18:3n-3 by 11.2% ($P = 0.03$) for the grain-fed steers but not for their forage-fed cohorts ($P = 0.20$; Fig. 2). Levels of 22:5n-3 and 22:6n-3 were higher for the grain-fed steers when they ate the non-treated flaxseed ($P \leq 0.02$), but levels of 22:5n-3 and 22:6n-3 were not affected by the type of flaxseed ingested for the forage-fed steers ($P \geq 0.09$). Total plasma n-3 FA concentrations were not influenced by type of flaxseed ingested regardless of basal diet ($P = 0.90$).

Forage-fed steers had slightly higher levels of phospholipid 22:5n-3 at the post-prandial time of blood collection ($P = 0.02$), but levels of phospholipids 22:6n-3 were higher at the pre-prandial time of blood collection for both basal diets ($P < 0.01$). Levels of phospholipids 18:3n-3 were not influenced by time of collection ($P \geq 0.67$).

Discussion

There are a number of treatment methods that have been used to treat flaxseed in order to reduce biohydrogenation.

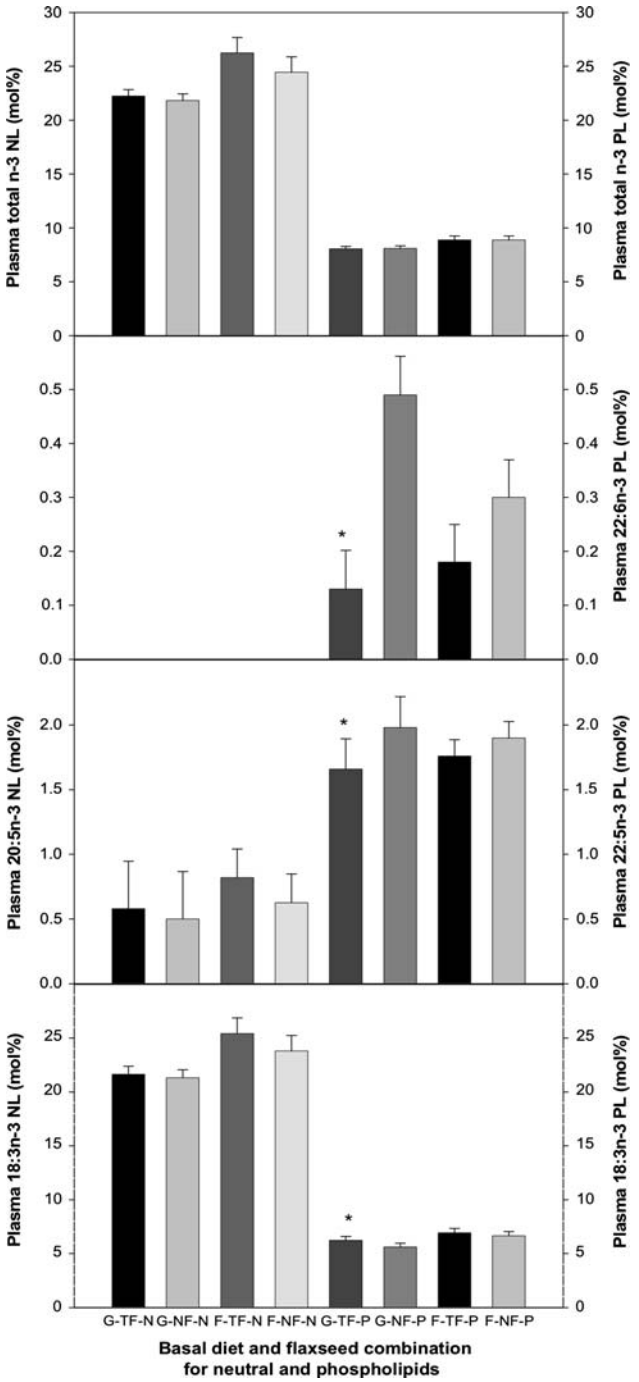

Fig. 2 Least square means and standard errors (*T-bars*) for mol% of 18:3n-3, 20:5n-3 and total n-3 (18:3n-3 and 20:5n-3 combined) in neutral (*N* or *NL*), and for mol% of 18:3n-3, 22:5n-3, 22:6n-3 and total n-3 (18:3n-3, 22:5n-3, and 22:6n-3 combined) in phospholipid (*P* or *PL*) in blood plasma of yearling Angus steers consuming either a grain-based diet (*G*) or a forage-based diet (*F*) and either tannin-treated ground flaxseed (*TF*) or non-treated ground flaxseed (*NF*). The three sets of four bars on the left side of the figure represent neutral lipid levels and the four sets of four bars on the right side of the figure represent phospholipid levels. The *asterisk* indicates significance from control $P < 0.05$

For instance, in lactating cows consuming a complex of a 1:1 (w/w) mixture of soybean and flaxseed oils mixed with a whey protein isolate gel, their mean 18:3n-3 level (g/100 g lipid) in the plasma triacylglycerol fraction was 4.9-fold higher than the same lactating cows consuming a 1:1 (w/w) mixture of soybean and flaxseed oils with their basal diet [25]. However, the practicality of this method is questionable. Additional treatment regimens include protecting 18:3n-3 by encapsulation of the n-3 containing feed in a matrix of formaldehyde-treated proteins [17]. Although this is likely the only practical method that may provide some protection for polyunsaturated FA (PUFA) from rumen microbial hydrogenation [15, 18], using formaldehyde in a production setting would undoubtedly meet with limited consumer acceptance. Thus, it is important to derive a useful method for protecting n-3 FA containing oil seeds from the n-3 FA reducing effects of ruminal biohydrogenation.

Although we found a significant protection in our fermentation experiment, a similar positive effect was not seen in vivo. Tannin-treated flaxseed did not increase 18:3n-3 or total n-3 FA levels in plasma neutral lipids from forage-fed or grain-fed steers. Because the rumen pH of cattle on a diet dominated by grains is acidic (pH $\leq$ 5.5), this ruminal environment was expected to reduce the effectiveness of condensed tannin as a protection treatment [36], however we did not expect a similar result with forage-fed cattle.

It is important to note that the seed coat of flaxseed may also provide a small amount of protection for 18:3n-3 from rumen microbes. However, beef muscle had more phospholipid 18:3n-3, 20:5n-3, and 22:5n-3 and more neutral lipid 18:3n-3 when cattle were fed ground versus whole flaxseed [10], suggesting that the seed coat is not an important factor in protecting 18:3n-3 from hydrogenation. Yet, when whole flaxseeds were fed to yearling steers, plasma 18:3n-3 content (weight % of total fatty acid methyl esters) increased 2.6-fold compared to that for steers not consuming flaxseed [13]. However, in the same experiment when flaxseed oil was continuously infused into the proximal duodenum of yearling steers, thus bypassing the rumen microbes, plasma 18:3n-3 content increased 5.7-fold compared to that of control steers [13]. Thus, it is important to get more 18:3n-3 into the duodenum and this indicates the potential for increasing plasma 18:3n-3 if a highly rumen-protective treatment can be found for flaxseed.

We observed in our in vitro trial that treating flaxseed with condensed tannin significantly reduced hydrogenation, with a loss of only 13% of the 18:3n-3 over 24 h versus a 43% loss for the untreated flaxseed. Despite this significant reduction we observed no increase in 18:3n-3 in plasma neutral lipid when cattle were fed tannin-treated flaxseed. There are several explanations for this disparity.

First, that absorption of fatty acids is influenced by the intestinal wall. However, since both post-ruminal infusions of flaxseed oil and flaxseed oil in a gel with a whey protein isolate have raised blood plasma levels of 18:3n-3, we doubt that fatty acid absorption through the intestinal wall accounts for this disparity. Second, ingestion and rumination in the in vivo trial versus lack of this chewing action in the in vitro trial may have allowed for greater hydrogenation of 18:3n-3 in the treated flaxseed for the in vivo trial. Third, the ratio of n-3 fatty acids to ruminal fluid differed between the fermentations in vitro and the feeding regimen in vivo. When fish oil is incubated for 24-h in vitro with sheep rumen fluid, only 10–15% of the n-3 FA were hydrogenated (when the ratio of fish oil to rumen fluid exceeded 5 mg/ml) [37]. They concluded that sheep rumen microbes showed a great capacity to hydrogenate 20:5n-3 and 22:6n-3 in fish oil when the concentration of oil was less than 1 mg per ml of rumen fluid. The ratio of flaxseed oil to rumen fluid in our in vitro trial was about 9 mg per ml of rumen fluid. We expect that the ratio in vivo was much lower. Recently, others have argued that unrealistically high levels of oil should be avoided in fermentations in vitro relative to the amount of substrate used [38]. They suggested that FA in the tested oil or oilseed should be about 3% of substrate DM. Perhaps, the higher ratio of treated flaxseed (and condensed tannin) to rumen fluid in the fermentations in vitro permitted a much greater suppression of microbial hydrogenation than in vivo with its lower level of tannin in the total ruminal mix. However, greatly increasing the amount of tannin-treated flaxseed fed to cattle is probably not a viable option given the cost of flaxseed and tannin, but it may be useful to evaluate other types of condensed tannin since they affect rumen microbes differently [39].

In summary, results from our cattle trial indicate that treating ground flaxseed with quebracho tannin is probably not an effective method for reducing hydrogenation of 18:3n-3 by ruminal microbes in cattle. A method that protects most if not all of the 18:3n-3 in flaxseed must be identified before beef with much higher levels of 18:3n-3 and its long chain derivatives can be produced.

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